# THE ENZYMOLOGY OF LYSINE BIOSYNTHESIS IN HIGHER PLANTS

## Complete localization of the regulatory enzyme dihydrodipicolinate synthase in the chloroplasts of spinach leaves

R. M. WALLSGROVE* and Mendel MAZELIS

*Department of Food Science and Technology, University of California, Davis, CA 95616, USA*

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### 1. Introduction

Lysine is synthesized in higher plants from aspartate, via the diaminopimelate pathway [1]. Intact isolated chloroplasts have been shown to synthesize all the aspartate-derived amino acids, including lysine, from exogenous [$^{14}$C]aspartate in the light [2]. The final enzyme of lysine biosynthesis, diaminopimelate decarboxylase (EC 4.1.1.20) has been found in *Vicia faba* chloroplasts [3] and shown to be completely localized in the chloroplasts in pea (*Pisum sativum*) leaves [4]. A number of other enzymes of the aspartate pathway have also been found in chloroplasts (reviewed [5]). In most instances the complete intracellular distributions have not been determined.

The first enzyme unique to lysine synthesis is dihydrodipicolinate synthase (EC 4.2.1.52), and this enzyme has been found in a variety of plant tissues [6]. So far there has been no report of the localization of this enzyme, though the results in [2] suggest that it is at least partly, if not completely, located in the chloroplast. As part of our investigation of the properties of spinach leaf dihydrodipicolinate synthase we have studied the distribution of the enzyme in mechanically prepared leaf homogenates and in protoplasts. This report clearly shows the chloroplast contains all of the enzyme present in the spinach leaf. Some of the difficulties and problems associated with the customary enzyme assay are also described as well as ameliorative modifications.

*Abbreviations:* ASA, aspartate-β-semialdehyde; *o*-ABA, *o*-aminobenzaldehyde

* Present address: Biochemistry Department, Rothamsted Experimental Station, Harpenden, AL5 2JQ, England

### 2. Materials and methods

Fresh spinach (*Spinacea oleracea* L.) was obtained from local markets. 'Cellulysin' and 'macerase' were purchased from Calbiochem. ASA was prepared by the method in [7], and the L-ASA produced was assayed using a partially purified homoserine dehydrogenase (EC 1.1.1.3) prepared from yeast [8]. *o*-ABA was synthesized by the method in [9] or purchased from Sigma Chemical Co. (MO). All other chemicals were obtained from commercial sources.

#### 2.1. *Chloroplast isolation*

Spinach leaf chloroplasts were prepared using the media and protocol of [10]. The washed chloroplast pellet was resuspended in buffer A which consisted of 50 mM K-phosphate (pH 8.0) containing 20 mM Na-pyruvate.

#### 2.2. *Protoplast isolation and fractionation*

Leaf protoplasts were isolated by a similar method to that described for pea leaves [11]. The lower epidermis was removed from washed spinach leaves, the leaf pieces floated on 1.5% Cellulysin, 0.5% Macerase and 0.5 M sorbitol at pH 5.5, and incubated at 30°C. After 3 h the protoplasts were collected and purified by flotation through 0.6 M sucrose. All the washing and purification media contained 50 mM Tricine · KOH (pH 8.0), 1 mM $MgCl_2$ and 10 mM Na-pyruvate. Protoplasts were broken by passage through a 20 μm nylon net, and a crude chloroplast fraction separated by centrifugation at 4000 × *g* for 1 min. This fraction was resuspended in buffer A. In some experiments a crude mitochondrial fraction was obtained by centrifuging the 4000 × *g* supernatant solution at 10 000 × *g* for 10 min.

2.3. *Dihydrodipicolinate synthase assay*

The assay used was a modified version of that in [12]. Before assay, all fractions were dialyzed overnight against buffer A. The assay mixture consisted of 100 mM Tris · HCl (pH 8.5), 100 mM Na-pyruvate, 5 mM L-ASA and up to 250 μl enzyme solution in 0.5 ml final vol. After incubation for 30–60 min at 30°C the reaction was stopped by the addition of 2 ml 0.22 M citrate/0.55 M $Na_2HPO_4$ solution at pH 5.0, containing 0.25 mg/ml *o*-ABA. The *o*-ABA was first dissolved in a minimum of ethanol. The color was allowed to develop for 2 h and after clarification by centrifugation, the $A_{520}$ was recorded. A reagent blank and enzyme blank lacking ASA were routinely included. A unit of enzyme activity is defined as a net absorbance increase of 0.001/min.

2.4. *Other assays*

Nitrite reductase was assayed by the method in [13], and cytochrome *c* oxidase as in [14]. Chlorophyll was determined in 95% ethanol using the extinction coefficients in [15].

## 3. Results

The assay of dihydrodipicolinate synthase in crude leaf extracts poses some problems. Formation of the chromophore between *o*-ABA and dihydrodipicolinate is inhibited or suppressed by thiol reagents (mercaptoethanol or dithiothreitol) and reducing agents (e.g., ascorbic acid), though these compounds appear to have little effect on enzyme activity per se (in preparation). In addition, the plant enzyme is strongly inhibited by low concentrations of L-lysine [6], and thus dialysis or gel filtration of crude extracts is essential before assay. With this precaution, enzyme activity is found in isolated spinach chloroplasts. When the distribution of the enzyme is compared to that of nitrite reductase (a wholly chloroplast-localized enzyme [11]) the proportion of the two enzymes in the chloroplast fraction is very similar (table 1). This suggests a similar intracellular distribution of the enzyme though the high degree of chloroplast breakage makes it difficult to be certain of this.

Use of isolated protoplasts makes it possible to recover a much greater proportion of intact chloroplasts and other organelles. Initial experiments with spinach protoplasts confirmed a chloroplast location for dihydrodipicolinate synthase. The activity of the supernatant (cytoplasmic) fraction was always extremely low, even after dialysis. It was found that a combination of heat treatment and dialysis was required to demonstrate the maximum activity in this fraction (table 2). It is clear that such endogenous inhibition is not present in the chloroplast fraction as none of the treatments had a significant effect on chloroplast enzyme activity.

Taking the maximum activity for each fraction, the enzyme distribution in lysed protoplast fractions is shown in table 3. The proportions of chlorophyll, nitrite reductase and dihydrodipicolinate synthase in these chloroplast fractions are very similar, indicating that chloroplast breakage was ≤20%, and that dihydrodipicolinate synthase is confined to the chloroplast in the intact cell. That this activity is not due to the mitochondrial contamination of the chloroplast fraction is shown by preparing a crude mitochondrial fraction as in section 2. This fraction contained >70% of the total cytochrome oxidase, but <5% of the dihydrodipicolinate synthase.

Table 1
Enzyme distribution in mechanically isolated spinach chloroplasts from leaf homogenates (% recovered activity)

| | Chlorophyll | Nitrite reductase | Dihydrodipicolinate synthase |
|---|---|---|---|
| 3000 × *g* pellet (chloroplasts) | 80 | 28.3 | 30.5 |
| Supernatant | 20 | 71.7 | 69.5 |

Recovered activities between 90–110% of the activity in the whole leaf homogenate

Table 2
Effect of dialysis and heat treatment on dihydrodipicolinate synthase in spinach protoplasts

| | Dihydrodipicolinate synthase (units/ml) | | | |
|---|---|---|---|---|
| | Undialyzed | Dialyzed | Heat treated | + Dialysis |
| Whole protoplast | 2.6 | 11.6 | 12 | 12 |
| Chloroplast | 25.8 | 27.5 | 21.6 | 23.8 |
| Supernatant | 0.2 | 0.48 | 0.13 | 1.6 |

Heat-treated sample heated at 60°C for 1 min, then rapidly cooled and centrifuged. Samples dialyzed versus buffer A for 18 h

Table 3
Enzyme distribution in spinach protoplast lysates (% recovered activity)

| | Chlorophyll | Nitrite reductase | Cytochrome *c* oxidase | Dihydrodipicolinate synthase |
|---|---|---|---|---|
| 4000 × *g* pellet | 83.3 | 79.5 | 28 | 86 |
| Supernatant | 16.7 | 20.5 | 72 | 14 |

Mean of 3 experiments. Recovered activities between 90–110% of activity in whole protoplasts

## 4. Discussion

The first enzyme unique to the biosynthesis of lysine and the last enzyme in the pathway have now been shown to be present only in the chloroplast in green leaves. Although the intermediate enzymes have never been demonstrated in plant tissues, the ability of isolated chloroplasts to synthesize lysine from aspartate indicates that they are also present in chloroplasts, and almost certainly located solely in the chloroplast. Therefore all lysine in the leaf must be synthesized in the chloroplast, and mechanisms presumably exist to transport lysine rapidly to other sites in the cell. It is conceivable that a small amount of lysine may be transported into the leaf, although studies of the nitrogen fraction in the phloem or xylem streams have never revealed much lysine to be present. Any accumulation of lysine will quickly shut down lysine synthesis, given the high sensitivity of dihydrodipicolinate synthase to feedback inhibition by this amino acid [6].

Chloroplasts also have the ability to synthesize the other aspartate-derived amino acids [2], the aromatic amino acids [16,17] and leucine [18], and there is no evidence for the synthesis of these amino acids outside the chloroplast (with the exception of methionine [19]). Although complete enzyme localization studies have not been carried out, it is tempting to speculate that the enzymes involved are wholly located in the chloroplast, and probably in the plastids of non-green tissues. This group of amino acids includes all those that are essential in the diet of non-ruminant animals, and this localization of amino acid biosynthesis could be of evolutionary significance in terms of the divergence between plants and animals. The presumed endosymbiosis of a photosynthetic prokaryote may have also endowed the primitive eukaryote with its only ability to synthesize these amino acids.

It will of course be necessary to substantiate the idea that all the enzymes involved are wholly chloroplast located, or at least that the pathways as a whole only exist in the chloroplast. Such localization studies also need to be done with non-green tissues to elucidate the role of the plastids in such tissues. It will also be of interest to see if any of the enzymes are coded for by the plastid genome.

So far, nearly all the work on the localization of the aspartate pathway has been carried out using $C_3$ plants – it remains to be seen whether the conclusions from these studies are also valid for $C_4$ plants.

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